REMARKS/ARGUMENTS

Claims 48, 49, 51-53, 55-59 and 66-81 are pending. Claims 1-47, 50, 54, and 60-65 have been cancelled without intending to abandon or to dedicate to the public any patentable subject matter.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner has rejected Claims 48, 49, 53, 55, 56, 66, 67, 73 and 74 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner states that the claims do not make clear "which expression levels" of genes are correlated with sensitivity or resistance to the EGFR inhibitor. Applicants understand the Examiner's remarks to require the recitation of the expression of a specific biomolecule associated with the gene expression of the recited genes.

Applicants have amended Claim 66 to recite the detection of the expression of polynucleotides of the recited genes and the comparison with the polynucleotide expression of the recited genes. Applicants submit that the pending claims, as amended, are sufficiently definite to meet the requirements of 35 U.S.C. § 112, second paragraph.

Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected Claims 48, 49, 53, 55-58, 66, 67, 73 and 74 under 35 U.S.C. § 112, first paragraph, as lacking enablement necessary for the skilled artisan to practice the invention commensurate in scope with the pending claims. The Examiner argues that while the claims are enabled for the evaluation of non-small cell lung cancer (NSCLC) tumors using tumor cells known to be resistant to gefitinib, the claims are not enabled for the evaluation of other tumor types using other EGFR inhibitors using any expression level. Solely to expedite prosecution, Applicants have amended the claims to focus on the detection of the polynucleotide expression level of the recited genetic biomarkers of EGFR inhibitor resistance and sensitivity. Thus, the pending claims are not directed to methods including the detection of polypetides.

In making the rejection for the lack of enablement for methods of evaluating a patient with a cancer other than NSCLC or for the administration of an EGFR inhibitor other than

gefitinib, it appears to be the Examiner's position that only those cancers and EGFR inhibitors tested and described in the Examples section of the specification are enabled. Applicants respectfully disagree. As stated in the guidance provided by section 2164.02 of the MPEP, compliance with the enablement requirement of 35 U.S.C.112, first paragraph, does not turn on whether an example is disclosed, and an applicant need not have actually reduced the invention to practice prior to filing. While the lack of a working example is a factor to be considered, Applicants need not describe all actual embodiments, and the presence of only one or no working example should never be the sole reason for rejecting claims as being broader than the enabling disclosure.

With respect to the recitation of cancers other than NSCLC, those skilled in the art of cancer treatment recognize that EGFR activation has been shown to play a role in the development of many cancers of epidermal origin. For example, Buck et al, *Inactivation of Akt by the epidermal growth factor receptor inhibitor erlotinib is mediated by HER-3 in pancreatic and colorectal tumor cell lines and contributes to erlotinib sensitivity*, Mol. Cancer Ther. 5(8):2051-59 (2006), a copy of which is enclosed herewith for the Examiner's convenience) shows the correlation between ErbB3 expression and EGFR inhibitor sensitivity. Thus, as shown by Buck et al., numerous cancers show an EGFR inhibitor sensitivity correlated with biomarker detection and the level of skill in the art is such that the claimed invention involves the use of tumor types known to show sensitivity to therapeutic intervention correlated with EGFR activity that may be evaluated by the detection of biomarker levels.

Similarly, with respect to the use of tumor cells that have a known sensitivity or resistance to an EGFR inhibitor other than gefitinib, Applicants submit that one of skill in the art of cancer chemotherapy understands that the inhibition of EGFR activity represents a common mechanism of action for all of the recited EGFR inhibitors, agonists thereof, or drugs having substantially similar biological activity as EGFR inhibitors and that a showing of resistance or sensitivity to agents that interact with this common mechanism is extended to all those therapeutic agents that share this common mechanism of action. Therefore Applicants submit that the pending claims directed to EGFR inhibitors, agonists thereof and drugs having a similar biological activity, are supported by the specification showing of sensitivity or resistance correlated with the example compound gefitinib, that shares the same mechanism of action as the

claimed family of compounds.

Applicants therefore submit that the pending claims are sufficiently supported and enabled in the specification to meet the requirements of 35 U.S.C. § 112, first paragraph.

Double Patenting

The Examiner has made a provisional rejection of Claims 48, 49, 53, 55-58, 66, 67, 73, 74 under the judicially-created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-15 of U.S. Patent Application Serial No. 11/781,946. There are currently no claims from either the instant application or co-pending Application No. 11/781,946. Therefore, Applicants will address this provisional rejection when allowable subject matter from these applications has been identified.

Based upon the foregoing, Applicants believe that all pending claims are in condition for allowance and such disposition is respectfully requested. In the event that a telephone conversation would further prosecution and/or expedite allowance, the Examiner is invited to contact the undersigned.

Respectfully submitted,
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Inactivation of Akt by the epidermal growth factor receptor inhibitor erlotinib is mediated by HER-3 in pancreatic and colorectal tumor cell lines and contributes to erlotinib sensitivity

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Abstract

Signaling through the receptor for epidermal growth factor receptor (EGFR) is frequently deregulated in solid tumors. Erlotinib (Tarceva, OSI-774, OSI Pharmaceuticals, Inc., Melville, NY) is a low molecular weight, orally bioavailable inhibitor of the EGFR that has been approved for both non–small cell lung cancer and pancreatic cancers. Previous studies have indicated that sensitivity to EGFR antagonists correlated with HER-3 signaling for non–small cell lung cancer. Herein, we have sought to understand the signaling pathways that mediate erlotinib sensitivity for pancreatic and colorectal cancers. In a panel of 12 pancreatic tumor cell lines, we find that EGFR is coexpressed with HER-3 in all cell lines sensitive to erlotinib but not in insensitive cell lines. Erlotinib can block HER-3 phosphorylation in these sensitive cell lines, suggesting that HER-3 is transactivated by EGFR. Knockdown of HER-3 in BxPC3, an erlotinib-sensitive pancreatic tumor cell line, results in inhibition of the phosphorylation for both Akt and S6 and is associated with a decrease in cell proliferation and reduced sensitivity to erlotinib. Therefore, EGFR transactivation of HER-3 mediates Akt signaling and can contribute to erlotinib sensitivity for pancreatic tumors. We extended our analysis to a panel of 13 colorectal tumor cell lines and find that, like pancreatic, HER-3 is coexpressed with EGFR in the most erlotinib-sensitive cell lines but not in erlotinib-insensitive cell lines. These studies suggest that HER-3 could be used as a biomarker to select patients who are most likely to respond to erlotinib therapy. [Mol Cancer Ther 2006;5(8):2051–9]

Introduction

The epidermal growth factor (EGF) receptor (EGFR) coordinates a variety of cellular activities, including the proliferation and migration of healthy cells in response to wounds (1, 2). This process is tightly regulated, and loss of restraint in EGF signaling contributes to uncontrolled cell proliferation and tumorigenesis. EGFR is one of four members of the HER family of cell surface receptor tyrosine kinases: HER-1 (ErbB1 or EGFR), HER-2 (ErbB2), HER-3 (ErbB3), and HER-4 (ErbB4; ref. 3). All members of this receptor family have the potential to mediate the deregulated signaling cascades that lead to cancer.

Erlotinib (Tarceva, OSI-774, OSI Pharmaceuticals, Inc.) is a low molecular weight, orally bioavailable inhibitor of EGFR and exhibits >100-fold selectivity for EGFR over other receptor tyrosine kinases, including PDGFR, insulin-like growth factor-I receptor, and HER-2 (4, 5). Erlotinib is clinically approved for the treatment of advanced non–small cell lung cancer (NSCLC; refs. 6, 7), and erlotinib, in combination with gemcitabine, has been recently clinically approved for nonresectable pancreatic cancer (8). Individual tumor cell lines exhibit varying degrees of sensitivity to EGFR inhibition (9–11). Therefore, identifying biomarkers for tumors that respond to erlotinib is important to enrich for patients who are likely to receive the most benefit from this targeted therapy. Previous reports had indicated that mutations in the kinase domain of EGFR were predictors of responsiveness and survival for EGFR inhibitor therapeutics (12, 13). However, further analysis of patients carrying these mutations revealed that they were more likely to perform well regardless of therapy received, and patients with wild-type EGFR still responded to erlotinib therapy and could receive survival benefit (7, 14). Therefore, although mutations might increase the likelihood of responding to EGFR inhibition, mutation status is not an indicator of overall survival benefit from EGFR inhibition for NSCLC. Moreover, other tumor types, including pancreatic and colorectal cancer, have been reported to respond to EGFR inhibitors, although mutations have not been found in these types of tumors (10, 12, 15). This questions the utility of employing EGFR mutation status to stratify erlotinib-sensitive patients and highlights the need for additional biomarkers of sensitivity to EGFR inhibitors for NSCLC as well as other tumor types.

Previous studies have shown that epithelial to mesenchymal transition (EMT) was a determinant of erlotinib sensitivity for NSCLC (15). Tumor cells that retained epithelial markers, including E-cadherin, were sensitive, whereas those that had undergone EMT and lost

E-cadherin while gaining markers of a mesenchymal phenotype, including Zeb1 and vimentin, were less sensitive (15). We have found that EMT is also a predictor of erlotinib sensitivity for pancreatic tumor cell lines.[3] Understanding the signaling cascades that mediate erlotinib sensitivity in tumor cells will allow us insights into how other targeted chemotherapeutic agents may be combined with erlotinib to enhance its effects.

EGFR is activated through ligand binding and dimerization (16). Ligand binding to the extracellular domains of EGFR induces conformational changes that promote dimerization with other EGFR monomers. Homodimerization results in transphosphorylation of tyrosine residues within the cytoplasmic domain of the receptor, promoting the intrinsic kinase activity of the receptor and providing a scaffold for several proteins, including adaptors and other kinases. EGFR can also heterodimerize with other members of the HER family, including HER-2 and HER-3 (17). HER-2 lacks a known ligand but can heterodimerize with EGFR, promoting phosphorylation and activation of the kinase activity of HER-2 (18). EGFR can also heterodimerize with HER-3 (19). As HER-3 lacks its own intrinsic kinase activity, it requires heterodimerization with either HER-2 or EGFR for phosphorylation (20, 21). Phosphorylated HER-3 acts as a scaffold to recruit signaling proteins, including phosphatidylinositol 3-kinase (PI3K). Therefore, EGFR may initiate cellular signaling cascades by itself or through its ability to transactivate other HER members.

Signaling proteins modulated by the EGFR include extracellular signal-regulated kinase (ERK) and Akt (2, 22). EGFR activation of Ras relays signals through the Raf-mitogen-activated protein kinase (MAPK)/ERK kinase-ERK pathway, culminating in regulation of cell cycle progression and proliferation. Alternately, EGFR activation of protein kinase C can promote ERK activation by directly phosphorylating Raf (23, 24). EGFR can also relay signals through PI3K to Akt. Akt directly phosphorylates and activates multiple antiapoptotic factors within the cell. In this manner, Akt activity mediates cell survival in the presence of stress (25, 26). Akt can also transmit signals through the mammalian target of rapamycin (mTOR)-S6 kinase-S6 pathway to affect cell proliferation (26–28). The ability of the EGFR inhibitors to down-regulate Akt activity has been reported previously to track with sensitivity to growth inhibition (9, 10). In a panel of six NSCLC tumor cell lines that were sensitive to the EGFR inhibitor gefitinib, Akt activity was mediated by HER-3, and HER-3 was shown to be expressed at a higher level in these sensitive cell lines compared with insensitive cell lines (10). HER-3 contains at least six binding sites for PI3K, and it is the most efficient member of the HER family to convey activation of PI3K to the Akt pathway (29, 30). In sensitive NSCLC tumor cell lines, Akt activity seems to be mediated by EGFR transactivation of HER-3. However, the effect of directly

modulating HER-3 signaling on the sensitivity of EGFR antagonists has not been determined. In addition, the role of HER-3 in mediating Akt activity and sensitivity to EGFR antagonists for other tumor types has not been established.

Herein, we sought to better understand the signaling events mediated by erlotinib in pancreatic tumor cells that were important for sensitivity. We determined the sensitivities of a panel of pancreatic and colorectal tumor cell lines to growth inhibition by erlotinib. EGFR was expressed in all cell lines, but EGFR expression alone did not track with erlotinib sensitivity. Of the four members of the HER family, only coexpression of EGFR with HER-3 tracked with sensitivity to erlotinib. A direct comparison of 34 cell lines, derived from pancreatic, colorectal, and NSCLC, revealed a strong correlation between coexpression of EGFR with HER-3 and sensitivity to growth inhibition by erlotinib.

We could detect phosphorylation of HER-3 in the four most sensitive pancreatic tumor cell lines but not in cell lines that were insensitive to erlotinib. We find that erlotinib could inhibit the phosphorylation of HER-3 in erlotinib-sensitive pancreatic cancer cells, indicating that EGFR can transactivate HER-3 in these cell lines. Further, we find that erlotinib could inhibit the Raf-MAPK/ERK kinase-ERK signaling cascade in both sensitive and insensitive pancreatic tumor cell lines, but inactivation of the Akt-mTOR-S6 cascade occurred only in cell lines that were sensitive to erlotinib. Using small interfering RNA (siRNA) to knockdown protein levels of HER-3 in an erlotinib-sensitive pancreatic cell line, we showed that this receptor mediates activation of the Akt-mTOR-S6 pathway. Knockdown of HER-3 decreased basal cell proliferation and reduced sensitivity to erlotinib by >3-fold. Therefore, the ability of erlotinib to inactivate HER-3 contributes to modulation of the Akt-mTOR-S6 pathway and growth inhibition by erlotinib. These data invite the use of HER-3 as a biomarker to select patients who are most likely to respond to erlotinib treatment and support further investigations into combining erlotinib with another targeted agent that inactivates the Akt-mTOR-S6 pathway in cell lines that do not express HER-3.

Materials and Methods
Cell Lines and Growth Inhibition Assays

The pancreatic cancer cell lines HPAC, CFPAC, BxPC3, Panc1, MiaPaca-2, A1165, Hs766T, SW1990, Capan-1, Capan-2, and HPAF-II were cultured in the appropriate American Type Culture Collection (Manassas, VA)–recommended supplemented medium. For growth inhibition assays, cells were plated and allowed to proliferate for 24 hours. After 24 hours, cells had reached ~ 15% confluency, at which time serial dilutions of erlotinib were added and the cells grown for a further 72 hours. Cell viability was assayed using the Cell TiterGlow reagent (Promega Corp., Madison, WI). Cell proliferation was assayed using a Bromodeoxyuridine ELISA Assay (Roche, Indianapolis, IN). For these experiments, the bromodeoxyuridine signal was normalized to total cell number.

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[3] Unpublished data.

Preparation of Lysates and Western Blotting

Cell extracts were prepared by detergent lysis [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS containing protease (Sigma, St. Louis, MO) and phosphatase (Sigma) inhibitor cocktails]. The soluble protein concentration was determined by micro-BSA assay (Pierce, Rockford IL). Protein immunodetection was done by electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose, incubation with antibody, and chemiluminescent second step detection (PicoWest; Pierce). The antibodies included EGFR, phosphorylated EGFR (Y1068), ErbB2, phosphorylated ErbB2, ErbB3, phosphorylated ErbB3, ErbB4, phosphorylated p42/p44, phosphorylated Akt (473), phosphorylated Akt

(308), total Akt, phosphorylated S6 (235/236), and total S6. With the exception of total ErbB3 (Santa Cruz Biotechnology, Santa Cruz, CA), all antibodies were obtained from Cell Signaling Technology (Danvers, MA).

For analysis of the effect of erlotinib on the phosphorylation of downstream signaling proteins, cell lines were grown to ~70% confluency, at which time erlotinib was added at the indicated concentration, and cells were incubated at 37°C for 2 hours. Where indicated, 10 ng/mL EGF ligand was added for 5 minutes. The medium was removed, cells were washed twice with PBS, and cells were lysed as described previously.

Taqman Assays

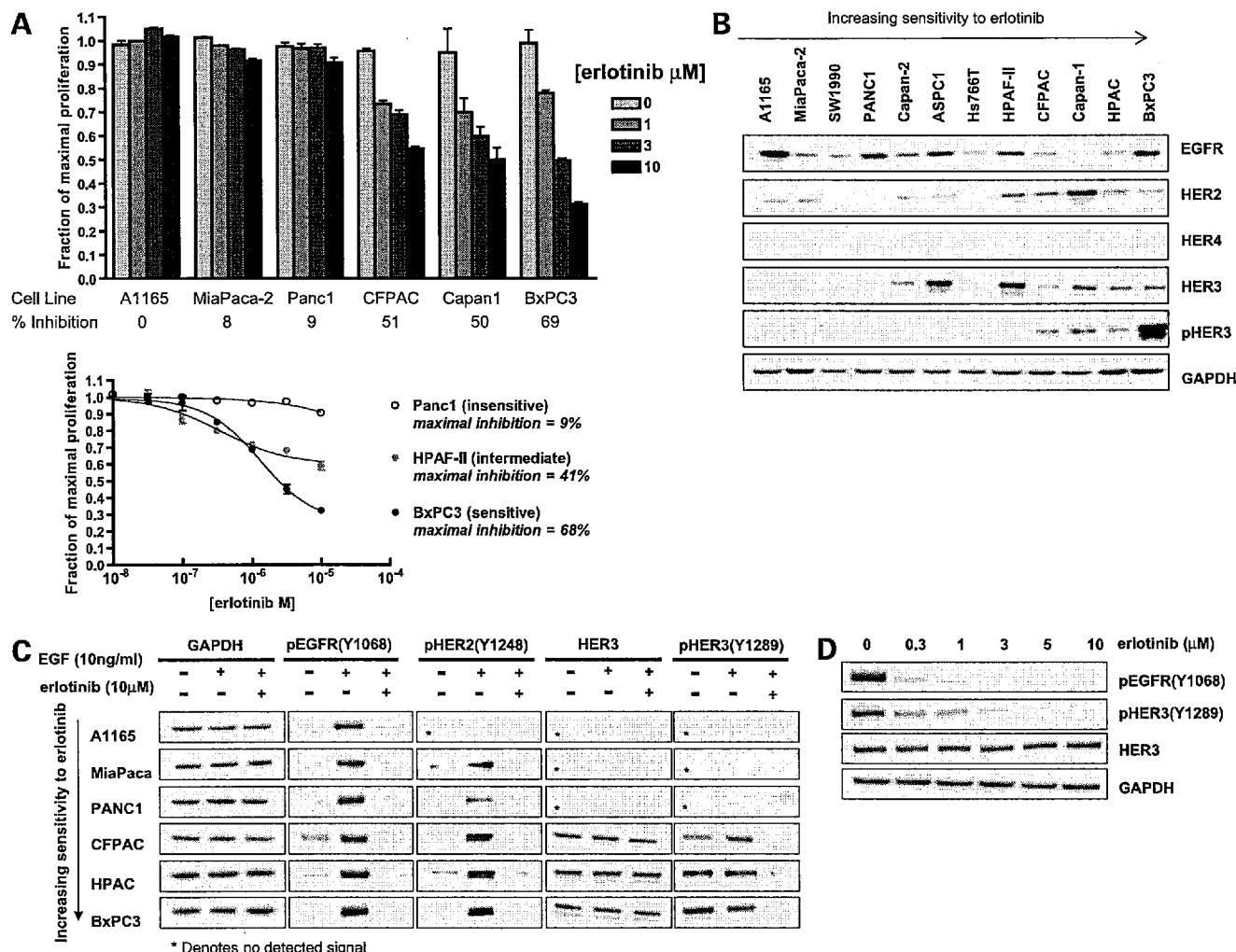Gene expression assays for HER-1, HER-2, HER-3, and HER-4 were obtained from Applied Biosystems (Foster

Figure 1. **A,** *top,* effects of varying concentrations of erlotinib (0, 1, 3, or 10 μmol/L) on the proliferation of three erlotinib-insensitive (A1165, MiaPaca-2, and Panc1) and three erlotinib-sensitive (CFPAC, Capan-1, and BxPC3) cell lines. Maximal percent inhibition of proliferation at 10 μmol/L erlotinib. *Bottom,* effects of varying concentrations of erlotinib on the growth of representative insensitive (Panc1), intermediate sensitive (HPAF-II), and sensitive (BxPC3) cells. **B,** protein expression levels of the four members of the HER receptor family in a panel of 12 pancreatic cell lines (ordered in increasing sensitivity to erlotinib). Levels of phosphorylated HER-3 (Y1289). **C,** effect of EGF and erlotinib on the phosphorylation of EGFR, HER-2, and HER-3 in a panel of three erlotinib-insensitive (A1165, MiaPaca-2, and Panc1) and three erlotinib-sensitive (CFPAC, HPAC, and BxPC3) cell lines. **D,** effect of varying concentrations of erlotinib, in the presence of 10 ng/mL EGF, on the phosphorylation of EGFR and HER-3 in BxPC3.

City, CA). Quantitation of relative gene expression was conducted as described by the manufacturer using 30 ng template. To determine relative expression across cell lines, amplification of the genes for the HER family members was compared with amplification of the gene for glyceralde-hyde-3-phosphate dehydrogenase.

Knockdown of HER-3 Using siRNA

The HER-3 siRNA was a Smart Pool siRNA from Dharmacon (Lafayette, CO) (NM_001982). Nonspecific silencing control siRNA was a siRNA duplex from Qiagen (Valencia, CA) (target sequence: sense UUCUCCGAACGUGUCAC-GUdTdT and antisense ACGUGACACGUUCGGAGAA-dTdT). Cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Cells ($n = 100,000$) were plated in six-well plates in DMEM containing 10% FCS, L-glutamine, and sodium pyruvate and cultured for 24 hours to ~40% confluency. The cells were then transfected with 100 nmol/L siRNA and whole protein extracts were prepared after siRNA transfections at the indicated time points. Twenty-four hours after transfection, cells were analyzed to determine the efficiency of transfection with a fluorescent oligonucleotide control from Invitrogen. Transfection efficiency was ~80%. Cells were incubated in the presence of the specific siRNA oligonucleotides for 24 to 72 hours before harvesting cells for protein extraction.

Results

We measured the sensitivities of 12 pancreatic cell lines to growth inhibition by erlotinib (Fig. 1A; Table 1). We chose maximal growth inhibition achieved by 10 μmol/L erlotinib as the criteria for ranking the sensitivities of cell lines. Maximal growth inhibition in vitro by 10 μmol/L erlotinib closely mirrors percent tumor growth inhibition in vivo for many reported cell lines; therefore, effects by 10 μmol/L erlotinib are biologically important (15). Moreover, clinical studies have well documented the pharmacokinetic variables of erlotinib at 150 mg (the recommended dose). Specifically, these data show that trough, peak plasma, and average steady-state concentrations of erlotinib reach 9.6, 15.1, and 11.0 μmol/L, respectively (31–33), for nonsmoking patients. Therefore, growth inhibitory effects observed by concentrations of erlotinib up to 10 μmol/L are not only biologically important but are clinically relevant. The cell lines in this panel displayed a spectrum of sensitivity, ranging from 2% to 69% maximal growth inhibition. The effects of 1, 3, and 10 μmol/L concentrations of erlotinib for a panel of three erlotinib-insensitive (A1165, MiaPaca-2, and Panc1) and three erlotinib-sensitive (CFPAC, Capan-1, and BxPC3) cell lines (Fig. 1A, top) and dose-response curves for representative insensitive (Panc1), intermediate sensitive (HPAF-II), and sensitive (BxPC3) cell lines (Fig. 1A, bottom) show a continuum of

Table 1. Summary of 12 pancreatic and 13 colorectal tumor cell lines and their sensitivity to erlotinib

Cell line	Derivation	K-ras status	% Inhibition (±5%)
Pancreatic			
A1165	Primary	Mutant	2
MiaPaca-2	Primary	Mutant	8
PANC1	Primary	Mutant	9
SW1990	Metastasis (spleen)	Mutant	9
AsPC1	Metastasis (ascites)	Mutant	22
Capan-2	Primary	Mutant	22
Hs766T	Metastasis (lymph node)	Mutant	37
HPAF-II	Metastasis (peritoneal)	Mutant	41
Capan-1	Metastasis (liver)	Mutant	50
CFPAC	Metastasis (liver)	Mutant	51
HPAC	Primary	Mutant	53
BxPC3	Primary	Wild-type	69
Colorectal			
RKO	Not described	Wild-type	10
HCT-116	Not described	Mutant	8
Colo205	Metastasis	Wild-type	10
HT-29	Not described	Wild-type	25
Colo201	Primary	Wild-type	36
WiDr	Not described	Wild-type	37
SW480	Primary	Mutant	44
CBS	Not described	Not determined	50
GEO	Not described	Not determined	50
FET	Not described	Not determined	51
DLD-1	Not described	Mutant	61
HCT-8	Not described	Mutant	64
HCT-15	Not described	Mutant	67

NOTE: Derivation of the cell line (primary or metastatic), mutational status of K-ras, and percentage maximal growth inhibition in response to 10 μmol/L erlotinib.

Table 2. Transcript levels of the four members of the HER receptor family in a panel of two erlotinib-insensitive (MiaPaca-2 and Panc1) and two erlotinib-sensitive (HPAC and BxPC3) cell lines

	HER-1	HER-2	HER-3	HER-4
MiaPaca-2	16,103	11,870	41	1
Panc1	40,482	512	28	45
HPAC	20,032	594	291	10
BxPC3	27,554	603	580	6

NOTE: Transcript levels were determined by quantitative PCR.

sensitivities for cell lines in this panel and show that growth inhibition by erlotinib is dose dependent. Cell lines in this pancreatic panel were derived from both primary tumors and metastatic sites, but the derivation of the cell line was not a predictor of erlotinib sensitivity (Table 1). K-ras is mutated in >90% of pancreatic tumors, and tumors that contain mutated K-ras have been proposed to be insensitive to EGFR inhibitors, as the MAPK pathway could be constitutively activated downstream of EGFR (34–36). However, in this group of 12 pancreatic cell lines, all but one has a mutation in K-ras, and 7 of 11 cell lines that express mutated K-ras show some sensitivity to growth inhibition by erlotinib (Table 1; ref. 37).

We measured the expression levels of the four members of the HER family in this panel of pancreatic cell lines (Fig. 1B). EGFR expression could be detected in all cell lines tested, but expression levels of EGFR alone were not sufficient to render sensitivity to growth inhibition by erlotinib. HER-2 was variably expressed, but levels did not track with sensitivity. Expression of HER-4 could not be detected in any cell line in the panel. Among the HER family members, coexpression of EGFR with HER-3 was the best predictor of erlotinib sensitivity. HER-3 was expressed in the four most sensitive cell lines but not in the four least sensitive cell lines. Accordingly, we could detect the phosphorylation of HER-3 only in the four most sensitive cell lines. BxPC3 showed the highest level of HER phosphorylation and was by far the most erlotinib-sensitive

pancreatic cell line. BxPC3 cells exhibited a maximal growth inhibition *in vitro* of 69% by 10 μmol/L erlotinib and showed a percent tumor growth inhibition of 63% in *in vivo* xenograft experiments (data not shown). This is in contrast to another cell line, CFPAC, which was less sensitive to erlotinib (maximal inhibition, 51%; percent tumor growth inhibition, 40%) and expressed much lower levels of pHER-3. Therefore, although there might not be a strong numerical correlation between pHER-3 levels and erlotinib sensitivity, there is likely a qualitative relationship. We compared the relative levels of mRNA for the HER family members in two sensitive (BxPC3 and HPAC) and two insensitive (MiaPaca-2 and Panc1) cell lines by quantitative PCR (Table 2). In all cell lines, mRNA levels of EGFR exceeded those of other HER family members by several fold; however, mRNA levels of HER-3 were on average 14-fold higher in the two sensitive cell lines compared with the two insensitive cell lines.

ErbB3 has no intrinsic kinase activity of its own, but it can become phosphorylated through heterodimerization with EGFR or HER-2 (20, 21). Previous studies have indicated that the EGFR inhibitor gefitinib could inhibit phosphorylation of HER-3 in three NSCLC cell lines that were sensitive to gefitinib (10). We wondered whether erlotinib could also regulate the activity of HER-3 in pancreatic tumor cell lines. We selected three sensitive and three insensitive cell lines for further investigation. Erlotinib could inhibit both basal (data not shown) and EGF (10 ng/mL)–stimulated EGFR activity in all cell lines, indicating that erlotinib could still bind to EGFR in the insensitive cell lines (Fig. 1C). We observed inactivation of pHER-2 in response to erlotinib in five of the cell lines, although this did not track with sensitivity. However, erlotinib could fully inhibit both basal (data not shown) and EGF-stimulated phosphorylation of HER-3 only in the three sensitive cell lines. Phosphorylation of HER-3 either did not occur or was below the level of detection in the three insensitive cell lines (Fig. 1C). The phosphorylation of HER-3 was not augmented with added EGF. Basal state phosphorylation of EGFR could be detected in all cell lines, and this could be inhibited by erlotinib (data not
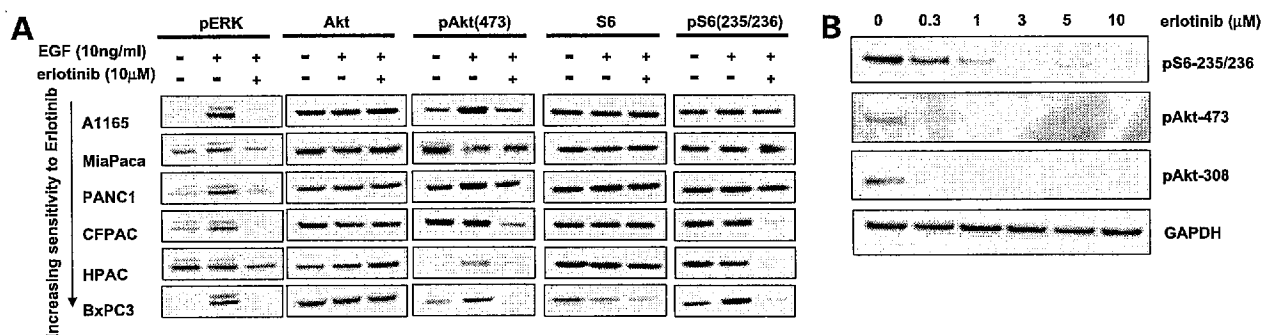

Figure 2. **A,** effect of EGF and erlotinib on the phosphorylation of ERK, Akt, and S6 in a panel of three erlotinib-insensitive (A1165, MiaPaca-2, and Panc1) and three erlotinib-sensitive (CFPAC, HPAC, BxPC3) cell lines. **B,** effect of varying concentrations of erlotinib, in the presence of 10 ng/mL EGF, on the phosphorylation of Akt (473), Akt (308), and S6 (235/236) in BxPC3.
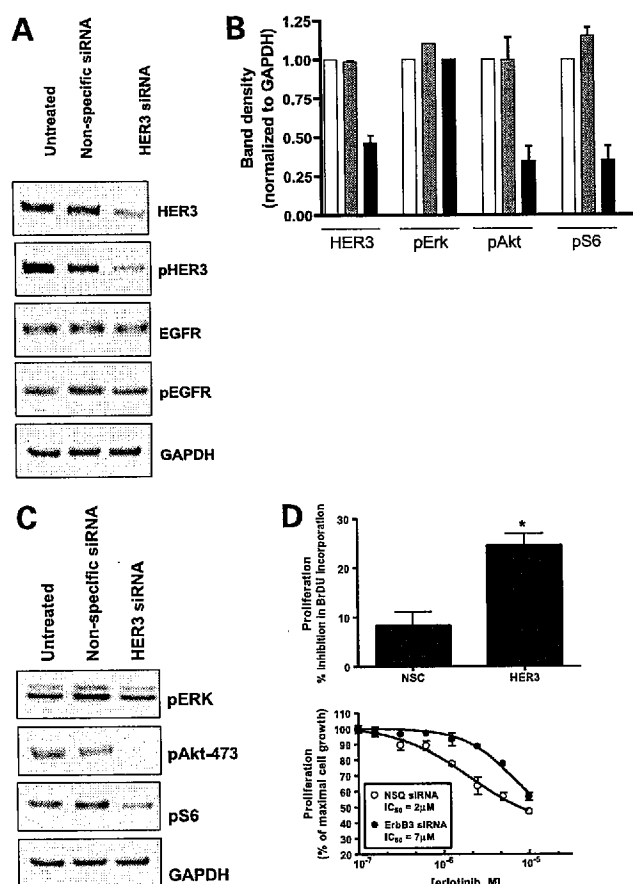
Figure 3. A, siRNA knockdown of HER-3 in the erlotinib-sensitive cell line BxPC3. Effect of HER-3 knockdown on the phosphorylation of HER-3 and EGFR. B, quantitative analysis of HER-3 protein expression and the phosphorylation states of ERK, Akt, and S6 from untreated control cells, cells treated with nonspecific control oligonucleotides, or cells treated with HER-3-specific oligonucleotides. C, effect of HER-3 knockdown on the phosphorylation of ERK, Akt (473), and S6. D, effect of HER-3 knockdown on the proliferation of BxPC3 cells. *, $P < 0.001$, statistically significant difference in proliferation between HER-3 knockdown cells and control cells (Student's t test). E, effect of varying concentrations of erlotinib on the proliferation of cells treated with nonspecific control oligonucleotides or oligonucleotides specific for HER-3. All siRNA experiments were repeated four times and yielded similar results.

shown). We suggest that, under basal conditions, EGFR is sufficiently activated to maximally phosphorylate HER-3. However, we cannot rule out the possibility that HER-2 participates in the regulation of HER-3 in these cells. Regardless of a direct or indirect effect, EGFR clearly participates in regulating HER-3 phosphorylation. The inhibition of EGFR and HER phosphorylation by erlotinib was dose dependent for BxPC3 (Fig. 1D). Band density analysis showed that 0.3 μmol/L erlotinib down-regulated 80% of EGFR-Y1068 phosphorylation and 70% of HER-3-Y1289 phosphorylation (data not shown). Therefore, signaling pathways important for the growth inhibition of erlotinib might be mediated by ErbB3.

We sought to better understand the signaling events downstream of EGFR and HER-3 that meditate sensitivity.

The Raf-MAPK/ERK kinase-ERK and Akt-mTOR-S6 pathways both lie downstream of EGFR and together control cell cycle progression and proliferation. EGFR activation of the Raf pathway, through the GTPase Ras or through protein kinase C, activates transcription factors important for cell cycle progression. Activation of mTOR facilitates the binding of mRNA to the ribosome and is a key regulator for translation of new proteins (38, 39). We find that erlotinib could inactivate pERK in both sensitive and insensitive cell lines (Fig. 2A). Inactivation of pERK by erlotinib occurred even in cell lines, such as A1165, Panc1, and CFPAC, which contain constitutively activating mutations in K-ras. This observation is consistent with previous reports for pancreatic tumor cell lines harboring K-ras mutations where ERK is not constitutively activated. Here, ERK activity might be mediated by EGFR activation of protein kinase C through phospholipase Cδ (40–43).

Erlotinib inactivation of pAkt and pS6 occurred only in those cell lines that were most sensitive (Fig. 2A). Although we observed some inhibition in EGF-stimulated Akt (473) phosphorylation for A1165, erlotinib could not achieve lower phosphorylation than basal conditions. The inactivation of these signaling proteins by erlotinib was dose dependent for BxPC3 (Fig. 2B).

HER-3 has been shown to mediate the activity of Akt. Here, HER-3 recruits PI3K, leading to activation of PDK1 and Akt. Akt can activate prosurvival factors to inhibit apoptosis. Alternatively, Akt can activate the mTOR-S6 kinase-S6 pathway to initiate translation of new proteins. We wondered whether the Akt and S6 activity in erlotinib-sensitive pancreatic cell lines was mediated by HER-3 signaling and if this pathway was directly involved in growth inhibition by erlotinib. In a previous report, HER-3 was shown to regulate the activity of Akt in NSCLC cell lines sensitive to gefitinib; however, the direct role of HER-3 on growth inhibition by gefitinib has not been shown (10). We used siRNA to knockdown the expression of HER-3 in BxPC3, the most sensitive in our panel of pancreatic tumor cell lines. A partial knockdown of HER-3 expression (~50–60%; Fig. 3A and B) was accompanied by a decrease in the phosphorylation of both Akt and S6 by ~65%, indicating that HER-3 lies upstream of this signaling pathway in BxPC3 (Fig. 3C). The HER-3 knockdown did not have a measurable effect on the phosphorylation of ERK. The lack of full inhibition of Akt and S6 phosphorylation is likely due to our lack of full inhibition of pHER-3 by siRNA. Erlotinib, which could inhibit Akt and S6 phosphorylation below levels of detection, was able to achieve complete inhibition of HER-3 phosphorylation. We find this partial knockdown of HER-3 expression decreased cell proliferation by ~17% compared with cells transfected with the nonspecific control oligonucleotide (Fig. 3D). If HER-3 is involved in mediating the growth inhibitory effects of erlotinib, then we would expect that the HER-3 knockdown cells should be less sensitive to erlotinib because their proliferation is less dependent on HER-3. We therefore compared the sensitivity to growth inhibition

by erlotinib in BxPC3 control cells with BXPC3 cells harboring the knocked down levels of HER-3. We observed a decrease in erlotinib sensitivity by >3-fold, from 2 to 7 μmol/L (Fig. 3E), indicating that HER-3 is important for the growth inhibitory effects of erlotinib. Collectively, these data indicate that the proliferation of BxPC3 cells is mediated in part by the ability of EGFR to transactivate HER-3.

HER-3 expression has been shown previously to track with sensitivity to EGFR inhibition in NSCLC (10, 11). We sought to determine if this might also be true of additional tumor types, so we measured the expression levels of the four members of the HER family in a panel of 13 cell lines derived from colorectal cancers. These colorectal tumor cell exhibited varying sensitivities to growth inhibition by erlotinib from 8% to 67% (Tables 1 and 2). As we observed for pancreatic tumor cell lines, EGFR could be detected in all, but expression levels did not track with sensitivity (Fig. 4A), indicating that expression of EGFR alone is not sufficient to mediate sensitivity. Expression levels of HER-2 also did not track with sensitivity, and the RKO cell line, which expressed the highest levels of HER-2, was the least sensitive. As with pancreatic cell lines, we find that coexpression of EGFR with HER-3 was the best predictor of sensitivity to erlotinib for colorectal tumor cell lines. When 34 cell lines derived from three tumor types (pancreatic, colon, and NSCLC) are directly compared, their sensitivity shows a strong correlation with HER-3 expression (Fig. 4B). The finding that the coexpression of EGFR with HER-3 correlates with erlotinib sensitivity globally across several tumor types invites its use as a biomarker to enrich for patients who are most likely to respond to this targeted agent.

Discussion

We have sought to determine the signaling events mediated by the EGFR inhibitor erlotinib that render pancreatic and colorectal tumor cells sensitive to growth inhibition. We find that coexpression of EGFR with HER-3 is a predictor of erlotinib sensitivity, and erlotinib could fully down-regulate HER-3 activity in sensitive pancreatic tumor cell lines. Therefore, proliferative signals in erlotinib-sensitive cell lines are likely mediated by EGFR trans-activation of HER-3. We speculate that the ability of erlotinib to inactivate HER-3 is likely through disruption of the EGFR/HER-3 heterodimer. However, we cannot rule out the possibility that HER-2 is coupling to HER-3 in these cells. Regardless of the mechanism, direct or indirect, the results clearly indicate that HER-3 regulation is EGFR dependent.

We analyzed signaling pathways downstream of EGFR and HER-3. Activity through the Raf-MAPK/ERK kinase-ERK pathway could be down-modulated by erlotinib in both sensitive and insensitive cell lines. All but one pancreatic tumor cell lines in our panel harbor constitutively activating mutations in K-ras; however, this did prevent erlotinib from inactivating ERK downstream. Previous studies have reported that ERK signaling is not constitutively activated

in pancreatic cell lines containing constitutively activated K-ras (41). Rather, ERK became phosphorylated only on exposure to growth factors and serum. Select cell lines containing activating K-ras repress constitutive ERK signaling through the increased expression of phosphatases, such as MAPK phosphatase-2 (42). EGFR activation of the phospholipase Cδ-protein kinase C-Raf pathway is one possible mechanism through which EGFR might activate the MAPK pathway in a Ras-independent manner (43). Alternatively, N-Ras, and not K-ras, might regulate the activity of the Raf-MAPK/ERK kinase-ERK pathway in select cell types. In Panc1 cells, which carry mutated K-ras, EGF leads to activation of the N-Ras-Raf-ERK cascade (44). This is consistent with the ability of erlotinib to inactivate ERK in Panc1. HCT-15, the colorectal tumor cell line that was most sensitive to erlotinib, also harbors a K-ras mutation. In HCT-15, we observed inhibition of ERK by erlotinib (data not shown). Therefore, ERK activity in pancreatic and colorectal tumor cells might be K-ras independent. These data also suggest that mutations in K-ras should not prevent patients from responding to erlotinib.
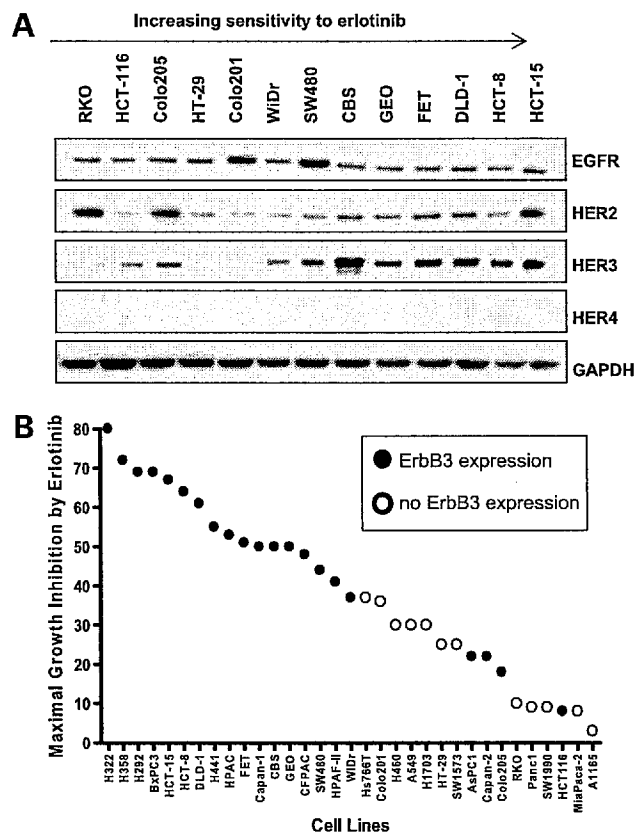

Figure 4. **A,** protein expression levels of the four members of the HER receptor family in a panel of 13 colorectal tumor cell lines. Cells lines are listed in order of increasing sensitivity to erlotinib. **B,** correlation of HER-3 expression with sensitivity to erlotinib in a panel of 34 cell lines derived from three tumor types (pancreatic, colorectal, and NSCLC). The maximal percentage growth inhibition with 10 μmol/L erlotinib was used as an evaluation of sensitivity. Cell lines are listed in order of decreasing sensitivity to erlotinib.
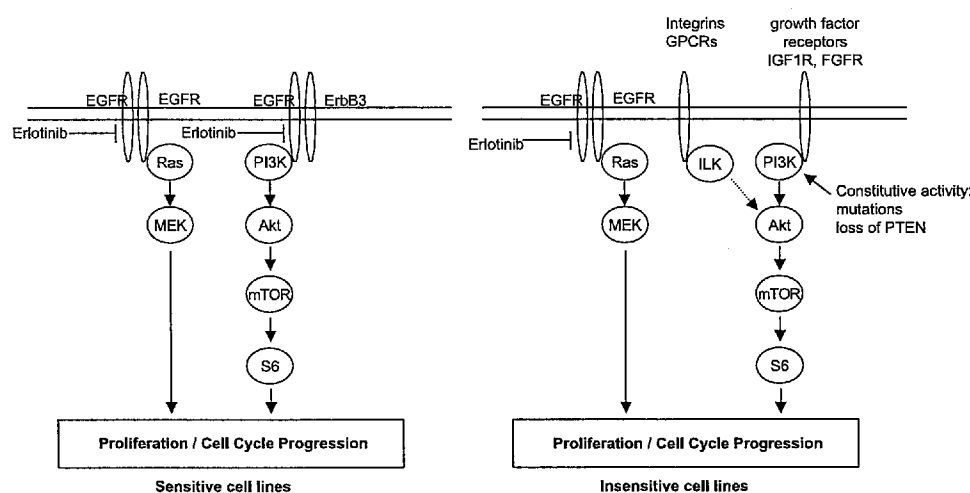
Figure 5. Proposed mechanism for proliferation and cell cycle progression in erlotinib-sensitive and erlotinib-insensitive cell lines. In sensitive cell lines, the Akt-mTOR-S6 pathway is mediated by the EGFR/HER-3 heterodimer. In insensitive cell lines, the Akt-mTOR-S6 pathway is mediated by several other potential mechanisms, including integrin and G protein-coupled receptor (*GPCR*) signaling, insulin-like growth factor-I receptor or fibroblast growth factor receptor growth factor signaling, or constitutive activity due to mutations.

The activities of Akt and S6 signaling proteins that lie upstream and downstream of mTOR, respectively, could be down-regulated by erlotinib only in sensitive cell lines. Previous studies have shown that HER-3 signaling can regulate the activity of the Akt-mTOR-S6 pathway (10, 19, 30). Here, we used siRNA to show that the activity of this pathway was mediated by HER-3 signals in the erlotinib-sensitive pancreatic tumor cell line BxPC3. HER-3 knockdown was accompanied by inhibition of both Akt and S6. We typically achieved a 50% to 60% knockdown of HER-3, and this likely explains why erlotinib, which can fully inhibit HER-3 phosphorylation, was able to achieve complete inhibition of Akt and S6, whereas the knockdown was only partial. We agree that we cannot completely rule out the possibility that some of the residual Akt and S6 phosphorylation is due to signaling other than directly through HER-3. For example, ERK signaling has been reported to affect S6 kinase signaling, and erlotinib does seem to inhibit ERK in a manner independent of HER-3. However, we find that the strong correlation between the extent of HER-3 inhibition (50–60%) and the inhibition of both pAkt and pS6 (65%) suggests that full Akt and S6 signaling may emanate from HER-3. Knockdown of HER-3 decreased basal cell proliferation by ~20% and lowered sensitivity to erlotinib by >3-fold. The knockdown of HER-3 was only partial (~60%), and this might explain why the inhibition of basal cell proliferation was modest. However, the remaining proliferative signals were less sensitive to erlotinib as indicated by the decrease in potency. These data show that the ability of erlotinib to block EGFR transactivation of HER-3 contributes to sensitivity to growth inhibition.

We suggest that, in tumor cells sensitive to erlotinib, cell cycle progression and proliferation rely on both EGFR homodimers and EGFR-HER-3 heterodimers. In insensitive cell lines that lack HER-3, the Akt-mTOR-S6 cascade likely is activated by alternative mechanisms. These might include other growth factor receptors (insulin-like growth factor-I receptor or fibroblast growth factor receptor) or integrin signaling (Fig. 5; ref. 45). Alternatively, this

pathway could be constitutively activated due to mutation. In the breast tumor cell line MDA-MB-468, which contains a mutation in PTEN, an endogenous inhibitor of PI3K, Akt signaling is constitutively activated (46). Restoring PTEN increases the sensitivity of this cell line to EGFR inhibition. Engineering the expression of HER-3 into erlotinib-insensitive cell lines would likely not change sensitivity. However, these observations suggest that combining erlotinib with another drug that targets insulin-like growth factor-I receptor, Akt, or mTOR may allow sensitization in cell lines that lack HER-3-mediated Akt activity.

Recent reports have shown that markers for epithelial and mesenchymal phenotypes can be used to distinguish sensitive and insensitive NSCLC cell lines (11). We have observed a similar correlation for pancreatic tumor cell lines.[3] Those cell lines that have undergone EMT are less sensitive to erlotinib. The expression of transcription factors, including Zeb1 and snail, which down-regulate the expression of epithelial cadherin (E-cadherin) and lead to EMT, have also been shown to be associated with decreased expression of HER-3 (47). We have observed a decrease in HER-3 expression when cells are treated with transforming growth factor-β to induce EMT.[3] Therefore, when cancer cells undergo EMT, their growth and survival may transition from HER-3 dependence to dependence on other growth factor pathways. The observation that differences in the expression of specific transcription factors likely control HER-3 expression levels in sensitive and insensitive tumor cell lines and not to changes in gene amplification is consistent with clinical observations (48).

The data presented here suggest that HER-3 might be a useful biomarker to select patients who are most likely to respond to the EGFR inhibitor erlotinib. Understanding how signaling pathways downstream of EGFR are regulated in sensitive and insensitive cell lines provides us with a means to rationally design combinations of molecular targeted therapeutics that are specifically tailored to a group of patients.

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